CLXXXVIII. THE DENATURATION OF PROTEINS.

VII. DENATURATION IN THE PRESENCE OF ALCOHOL.

BY NORMAN BOOTH.

*From the Muspratt Laboratory of Physical and Electro-chemistry,
University of Liverpool.*

ALTHOUGH it has been known for many years that the addition of alcohol to an aqueous solution of a denaturable protein accelerates the rate of denaturation, relatively little quantitative work seems to have been carried out in connection with the process. For this reason a systematic study of denaturation in a number of alcohol-water mixtures should be of value, more particularly as the conditions involve alterations in such factors as hydration and dielectric capacity of much greater magnitude than can be realised in the case of denaturation in aqueous solutions containing dilute acids, bases or salts.

In the present paper the investigation is confined to a study of the effect of alcohol upon heat denaturation, that is on denaturation in the neighbourhood of the neutrality point of water.

The protein used was haemoglobin, prepared from fresh defibrinated ox blood following the method employed by Lewis [1926] and Cubin [1929]. A method similar to that employed by these workers was adopted for determining the increase in denaturation with time. The absolute alcohol employed was digested with activated quicklime (prepared by roasting marble at 800–1000° in an electric oven for some hours), and distilled several times. The p_H of the solutions was determined by means of the quinhydrone electrode following the technique of Corran and Lewis [1924]. In using this method for determining the p_H, the usual "constant" in the E.M.F. expression cannot be employed in the calculations since the solubility of the quinhydrone varies with the composition of the alcohol-water mixture. A different "constant" must therefore be used for each alcohol concentration. In the present work these "constants" were obtained by extrapolating the values found by Millet [1927]. No change in p_H was observed during the course of the denaturation process, which agrees with results obtained for this protein in the absence of alcohol.

The concentration of alcohol was varied from 2 to 30 vols. % and the temperature at which the reaction was investigated was varied from 30° to

60·5°. The method of damping the reaction was by very rapid cooling in an ice-bath. Owing to the possibility of denaturation occurring at room temperature it was necessary to keep the solutions in the ice-bath except for the filtration and estimation processes which were carried out as rapidly as possible.

The results are given in Tables I to V. Satisfactory unimolecular velocity constants were obtained in all cases. Consequently the mean values alone are given. The unit of time employed is the second.

Table I.

Denaturation in 2 % alcohol

	p_H	$k \times 10^4$
(a) 60·5°	6·00	5·06
	6·33	3·23
	6·59	2·46
	6·76	2·28
	7·11	3·30
	7·25	3·79
(b) 55°	5·49	2·03
	6·01	0·60
	6·46	0·31
	6·62	0·28
	7·33	0·72
	7·41	0·84

Table II.

Denaturation in 4 % alcohol

	p_H	$k \times 10^4$
(a) 60·5°	5·67	7·16
	5·92	6·72
	6·07	5·09
	6·36	4·44
	6·48	4·81
	6·76	6·41
	7·08	8·22
(b) 55°	5·43	3·12
	5·99	0·84
	6·17	0·63
	6·28	0·51
	6·58	0·62
	7·06	1·45
	7·74	2·78

Table III.

Denaturation in 10 % alcohol at 55°

p_H	$k \times 10^4$
5·36	10·6
5·78	5·32
6·04	3·46
6·21	3·26
6·35	3·82
6·52	4·24
7·00	6·40

Table IV.

Denaturation in 20 % alcohol

	p_H	$k \times 10^4$
(a) 45°	5·19	18·6
	5·50	7·88
	6·01	4·56
	6·57	4·52
	6·78	5·44
	7·18	9·99
(b) 40°	5·54	0·52
	5·88	0·33
	6·09	0·27
	6·27	0·27
	6·58	0·35
	6·71	0·43
	7·08	0·72

Table V.

Denaturation in 30 % alcoho

	p_H	$k \times 10^4$
(a) 33°	5·82	13·2
	5·90	8·40
	6·04	4·02
	6·10	4·26
	6·32	5·24
	6·43	6·28
	6·75	8·68
(b) 30°	5·74	1·98
	6·04	0·60
	6·14	0·67
	6·36	0·84
	6·50	0·95

The general effect produced by alcohol is illustrated in Fig. 1 which for the sake of clearness gives only a selection from the data recorded, namely data from Tables I, II, III and V.
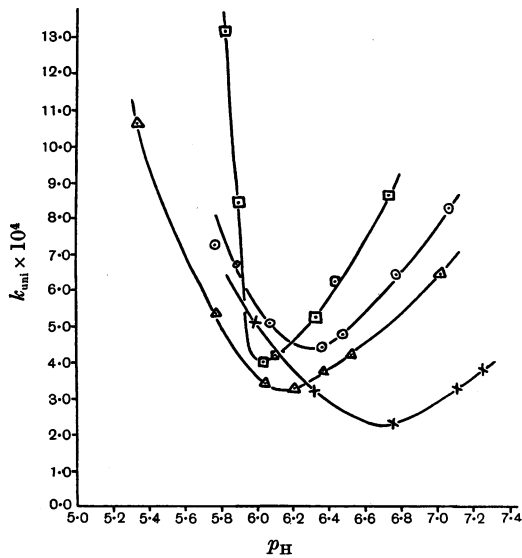

p_H.

Fig. 1.

The denaturation of haemoglobin in the presence of alcohol.

— × — × — represents denaturation in 2 % alcohol at 60·5°.
—⊙—⊙—   ,,   ,,   4 %   ,,   60·5°.
—△—△—   ,,   ,,   10 %   ,,   55°.
—▣—▣—   ,,   ,,   30 %   ,,   33°.

From the above results three facts of importance emerge. In the first place the speed of denaturation is increased by the presence of small amounts of alcohol. This is seen from Table VI, in which the values of the velocity constant in the presence of alcohol are compared with those in the absence of alcohol. The values quoted in the presence of alcohol are taken from the curves obtained by plotting the observed results. The results in the absence of alcohol at 60·5° were obtained by Lewis [1926]. Those given for the temperatures lower than 60·5 and in the absence of alcohol have been calculated on the

Table VI. *The variation in velocity constant with alcohol content.*

p_H	$k \times 10^4$ at 60·5°			$k \times 10^4$ at 55°			
	Water	2 % alc.	4 % alc.	Water	2 % alc.	4 % alc.	10 % alc.
6·0	3·55	5·06	5·70	0·46	0·60	0·84	3·75
6·5	1·53	2·69	4·88	0·20	0·30	0·52	4·24
7·0	1·03	2·85	7·72	0·14	0·37	1·30	6·40

p_H	$k \times 10^6$ at 45°		$k \times 10^7$ at 40°		$k \times 10^6$ at 33°		$k \times 10^7$ at 30°	
	Water	20 % alc.	Water	20 % alc.	Water	30 % alc.	Water	30 % alc.
6·0	0·97	456·0	1·27	291·0	0·0066	404·0	0·0179	600·0
6·5	0·42	420·0	0·44	322·0	0·0029	655·0	0·0077	980·0
7·0	0·28	720·0	0·27	643·0	—	—	—	—

basis that the critical increment between the p_H values of 6·0 and 7·0 is 80,000 cals. for haemoglobin.

The second fact which emerges from Tables I to V is that the presence of alcohol causes the minimum point in the p_H-velocity curve to shift from the neutrality point of water (p_H 6·99) to the acid side. This shift is considerable with small amounts of alcohol and increases slowly with further addition of alcohol. This is shown in Table VII.

Table VII. *Shift of the minimum point with alcohol content.*

Alcohol content in cc. per 100 cc. total volume	p_H of minimum point
0	6·99
2	6·70
4	6·40
10	6·20
20	6·16
30	6·01

The third fact which emerges from the results obtained is that the critical increment of the denaturation process increases with increasing alcohol concentration. This is shown by Table VIII. The values of the critical increment were calculated in the usual way, and hold for the whole of the p_H range investigated.

Table VIII. *Variation in critical increment with alcohol content.*

Alcohol content in cc. per 100 cc. total volume	Critical increment (cals.)
0	80,000
2	83,000
4	85,000
20	105,000
30	117,000

The increase in critical increment in 2 and 4 % alcohol solutions is so small that it might quite easily be attributed to the experimental error, but the increase in the case of 20 and 30 % alcohol solutions is far too large to be explained in this way.

DISCUSSION.

It will be noted that the critical increment (obtained in the ordinary way) increases with alcohol content whilst at the same time the velocity of denaturation likewise increases. This is contrary to what would be expected on the basis of statistical theory as applied to chemical reactions. The origin of the discrepancy must lie in the fact that the influence of temperature is not wholly operative in the ordinary sense, namely, in increasing the probability of rupture of a certain number of bonds which remain essentially of the same type and character over a range of temperature. Thus an increase in temperature may not only increase the probability of rupture, in the usual statistical sense, of the chemical bonds involved in denaturation but likewise may

either release, *i.e.* render available, a number of such bonds or cause a diminution in cohesion on the part of the colloidal unit as a whole. In other words the influence of temperature may be at least twofold. If this be the case the observed critical increment would be a composite quantity and could not be treated without further analysis in the usual statistical manner. It is hoped to examine this point in a subsequent publication from this Laboratory.

As regards the variation of the critical increment with alcohol content the following suggestion may be made. The dielectric constant of water is 81 at 20°, that of pure alcohol is 26, and that of an alcohol-water mixture lies between these two values. One result of the fall in dielectric constant on adding alcohol to the medium will be an increased difficulty in breaking down a bond, particularly if it be dipolar in nature. It seems possible that the increased critical increment may be due to this cause. If E_w is the critical increment in water and E the observed critical increment in a given alcohol-water mixture whose dielectric constant is D, then if D_w is the dielectric constant of water, we should expect on the simplest grounds that

$$E = E_w \cdot \frac{D_w}{D},$$

or
$$ED = E_w D_w = \text{constant}.$$

By interpolation of the data given in the International Critical Tables, Vol. 6, p. 98, the values of the dielectric constant at 20° for varying alcohol concentration are obtained. Using the equation

$$D_t = D_\theta \left[1 - \alpha \left(t - \theta\right)\right],$$

where D_t and D_θ are the values of the dielectric constant at the temperatures t and θ respectively, and α is a coefficient dependent only on the medium, the value of α for pure alcohol was calculated from the results of Rimbach and Weitzel [1912]. Since the value of α so obtained was found to be near to that for water, it was assumed that the values for alcohol-water mixtures were situated linearly between these two values, and these were calculated accordingly. Employing the values of α so obtained the dielectric constant was corrected to the temperature at which the critical increment was measured, in the case of each alcohol concentration. The values of $E \times D$ were then calculated and are given in Table IX, in the column headed $E \times D_{\text{corr}}$.

Table IX.

Alcohol volume %	E cals.	$D_{20°}$	$E \times D_{20°}$ $\times 10^{-6}$	D corrected for temp.	$E \times D_{\text{corr}}$ $\times 10^{-6}$	Wt % water (C_w)	$E \times D \times C_w$ $\times 10^{-8}$
0	80,000	81·0	6·48	66·2	5·30	100·0	5·297
2	83,000	79·5	6·60	64·9	5·39	98·42	5·302
4	85,000	78·2	6·57	63·7	5·41	96·83	5·242
20	105,000	69·3	7·28	62·6	6·57	83·85	5·512
30	117,000	64·0	7·49	60·8	7·11	75·47	5·369

It is evident that the product $E \times D$ rises steadily with increase in E. In other words the value to be ascribed to D to maintain the product ED con-

stant would be one which would fall more rapidly with increase in alcohol content than is indicated by the values in Table VIII. Owing to the molecules of the medium being almost certainly present in an oriented unimolecular layer [Svedberg and Nichols, 1927] the dielectric constant of the actual medium in which the denaturation reaction occurs probably has a value of the order of 7 in pure aqueous solution [Rideal, 1930].

Correspondingly reduced values should have been employed in the calculation of the product $E \times D$.

It will be evident however that simple proportionality between change in dielectric capacity of the surface layer and that of the bulk of the solution in the case of alcohol-water mixtures is only to be anticipated if the surface adsorbability of the water and alcohol molecules were the same; a very unlikely assumption. If alcohol molecules are preferentially adsorbed, for example, the value to be ascribed to D should fall more rapidly than is indicated by the values employed in Table VIII. As a matter of fact it is found that the empirical relation $E \times D \times C_w$ is a constant, as is indicated by the final column of Table VIII, where C_w is the mass of water present in each alcohol-water mixture.

SUMMARY.

1. The speed of denaturation of haemoglobin has been determined in the presence of varying amounts of alcohol. The alcohol content was varied from 0 to 30 vols. %, the temperature from 30° to 60·5°, and the p_H from 5·19 to 7·74.

2. Unimolecular velocity constants were obtained throughout, and these constants increased in magnitude with increasing alcohol content under otherwise similar conditions.

3. It was found that the minimum point on the p_H-velocity curve was shifted considerably from the neutrality point of water to the acid side by addition of alcohol. The curve was no longer symmetrical about the minimum point, being more abrupt on the side of lower p_H values than on that of greater p_H values.

4. The critical increment of activation was found to increase appreciably with the amount of alcohol present. It was found that the magnitude of the critical increment was inversely proportional both to the dielectric constant of the medium and to the percentage by weight of water present.

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